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meeting report

The 30th anniversary of quasispecies

Meeting on 'Quasispecies: past, present and future'

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The meeting on 'Quasispecies: past, present and future' took place between 17 and 18 November 2008, in Barcelona, Spain, and was organized by J. Gómez, C. López-Galíndez, M.A. Martínez & A. Mas.

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Introduction

A meeting was held in Barcelona, Spain, in November 2008 to celebrate the 30th anniversary of the publication of the article that described the extensive genetic heterogeneity of bacteriophage Qβ (Domingo *et al*, 1978), which is considered to mark the beginning of experimental studies on viral quasispecies.

This meeting was held at the impressive fifteenth century building of the ancient Hospital de la Santa Creu in the old town of Barcelona, which is now the headquarters of the Institut d'Estudis Catalans, and was attended by C. Weissmann (Jupiter, FL, USA), M. Billeter (Zurich, Switzerland) and E. Domingo (Madrid, Spain), who were three early protagonists of the phage Qβ work at the University of Zurich in the 1970s. Several speakers presented their results on the theoretical aspects of the population dynamics of cells and viruses, the clinical implications of quasispecies, and extensions of the quasispecies concept to cellular genes and prions. The meeting was introduced by A. Mas (Albacete, Spain), who reflected on the increasing impact that quasispecies have had in the scientific literature over the past three decades, and quoted some of the key references on viral quasispecies (Martell *et al*, 1992; Meyerhans *et al*, 1989; Nájera *et al*, 1995; Vignuzzi *et al*, 2006; for a historical review of the impact of quasispecies in virology, see Holland, 2006). The scientific presentations were opened by Weissmann and Domingo, who were the last and first authors of the 1978 paper, respectively. Their talks conveyed the scientific atmosphere of the 1970s—when molecular biology was carried out with few recombinant-DNA techniques—to a young audience. Nucleic-acid sequencing was in its infancy and PCR was yet to be invented. Nevertheless, the Weissmann team pioneered a site-directed mutagenesis approach by exploiting the unique replicative properties of Qβ replicase *in vitro*, which allowed the introduction of mutagenic nucleotide analogues at preselected positions of the minus-strand Qβ RNA. Upon replication, the minus strands were copied into wild-type and mutant versions of the infectious plus strands. This research represented the birth of 'reverse genetics', which is today a much more facile undertaking. Unexpectedly, an extracistronic mutant constructed by Domingo was viable; however, it displayed a selective disadvantage relative to wild-type Qβ during both the replication of the RNA *in vitro* and the multiplication of the virus *in vivo*. Reversion and competition experiments with this mutant and the wild-type virus allowed the calculation of the mutation rate for a specific base transition as 1×10^{-4} substitutions per nucleotide copied, which is in line with later measurements of other RNA viruses (Batschelet *et al*, 1976; Domingo *et al*, 1976; Drake & Holland, 1999).

The determination of a mutation rate was only one side of the Qβ story in Zurich. The other side emerged in the course of control experiments—which are both necessary and frequently revealing. During the verification that a site-directed mutant had indeed been produced by site-directed mutagenesis—and was not present as a spontaneous mutant—the intrinsic sequence heterogeneity of

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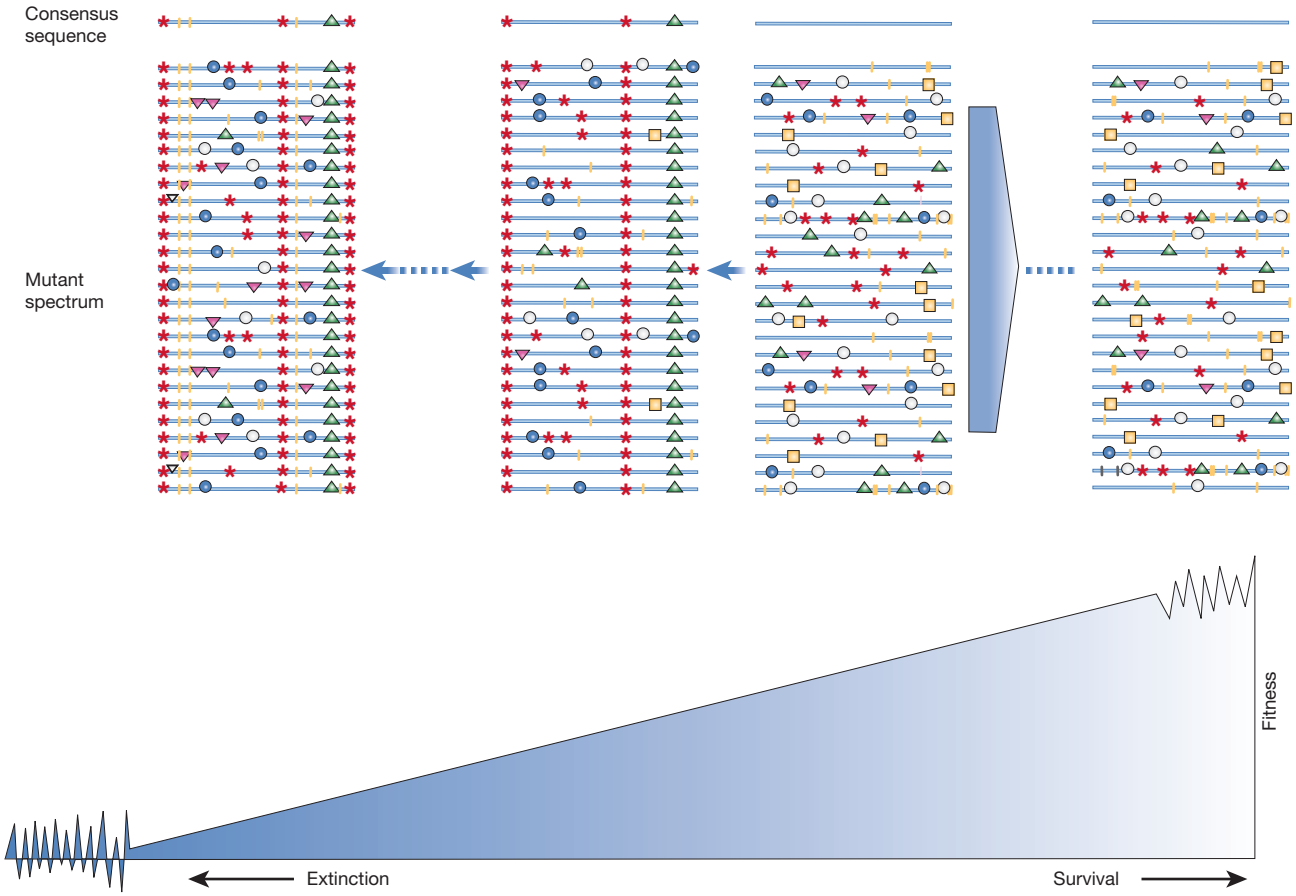

Fig 1 | Schematic of viral quasispecies and fitness variations. Mutant distributions—here, genomes are shown as lines and mutations are depicted as symbols on the lines—can be subjected to large population passages (large arrow on the right) that result in fitness gain. Repeated bottleneck events (small arrows) result in fitness decrease. Based on data reviewed in Domingo *et al* (2006).

Qβ populations was discovered. D. Sabo—who sadly passed away before the paper was published in *Cell* (Domingo *et al*, 1978)—and Domingo showed that phage clones, purified by isolation of the virus from individual plaques formed on *Escherichia coli* monolayers, rapidly developed into complex mixtures of variants. This spectacular diversification was uncovered by patiently labelling hundreds of phage clones with ^{32}P-phosphate—which would be unthinkable today—subjecting the RNA to digestion with ribonuclease T_1, and resolving the products by a two-dimensional gel-electrophoresis system that had been developed by R. de Wachter and W. Fiers in Belgium. The position of the oligonucleotides was highly sensitive to point mutations, which could then be confirmed by further ribonuclease-digestion analysis of the eluted oligonucleotides. The result of these analyses was inescapable, as written in the paper: "the phage population cannot be described as a defined unique structure, but rather as a weighted average of a large number of different individual sequences". The estimate was that, on average, each Qβ RNA genome differed by 1.6 mutations from the average or consensus sequence of the parental population. Quasispecies were mentioned only briefly in the discussion section of the paper as a personal communication from M. Eigen, who had heard about the Qβ experiments from Weissmann at the renowned Max-Planck Institute meeting in Klosters, Switzerland, in 1978.

The concept of quasispecies cannot be considered a modification of the concept of biological species. In the founding theory, the term quasispecies was applied to mutant distributions of primitive replicating entities, to emphasize the fact that they consisted of an ensemble of non-identical molecules, rather than a single molecularly-defined species. In its initial formulation, the quasispecies theory referred to steady-state mutant distributions of infinite size (Eigen & Schuster, 1979). Various extensions of this theory have been developed to describe finite distributions of genomes in variable-fitness landscapes, which fit the characteristics of viral populations (Fig 1; Sidebar A; reviewed in Domingo *et al*, 2006).

Viral quasispecies

The results obtained with the Qβ phage came as a surprise at a time when everyone was fiercely pursuing the sequence of 'their' virus. However, sequence heterogeneity was soon documented for FMDV (foot-and-mouth-disease virus) by Domingo and colleagues (Domingo *et al*, 1980; Sobrino *et al*, 1983), and for VSV (vesicular stomatitis virus) by J. J. Holland and colleagues (Holland

Sidebar A | Basic concepts of the quasispecies theory

Viral quasispecies: Dynamic distributions of genomes that are subjected to genetic variation, competition and selection, and act as a unit of selection.

Dominant genome: The genome that is more abundant in a mutant distribution, also known as a mutant spectrum.

Fitness: The relative replication capacity of a virus measured in a defined environment in a growth-competition assay with a reference clone.

Competitive exclusion: A principle of population genetics that states that, in the absence of niche differentiation, one competing species will always outcompete the other. In virology, this refers to the exclusion of one of two viruses or two viral mutants that compete for the same resources.

Red Queen dynamics: A hypothesis that proposes that an evolutionary system requires continuing development in order to maintain its fitness relative to coevolving systems. In virology, it has been adopted to describe the continuous increase of fitness of viral mutants engaged in competitive replication. In the words of L. Van Valen, "No species can ever win and new adversaries grinningly replace the losers", which is a reference to the words of the Red Queen in Lewis Carroll's *Through the Looking Glass* stating that "... it takes all the running you can do to keep in the same place".

Contingent neutrality: A relationship of neutrality between two mutant genomes that is dependent on a specific set of environmental conditions.

Molecular memory: The presence, in a viral quasispecies, of a class of minority genomes at frequencies higher than expected from the basal mutation rate that reflect those genomes that were dominant at an earlier stage of the same evolutionary lineage.

Error threshold: A critical average error rate above which the information encoded by a genetic system cannot be maintained.

Error catastrophe: Loss of infectivity owing to the accumulation of excess mutations in a virus. This is an extension of the concept of violation of the error threshold, derived from the quasispecies theory, to viruses. Owing to the differences between the theoretical concept and the events underlying virus extinction, the term lethal mutagenesis is often used instead.

et al, 1982). The studies of Holland over the following decades were discussed at the meeting by Domingo, who highlighted, as major contributions from the Holland laboratory at the University of California, San Diego (CA, USA), the first experimental evidence of error catastrophe—also known as lethal mutagenesis—and the suppression of a high-fitness mutant by a mutant spectrum of inferior fitness, the first quantification of viral fitness and evidence of contingent neutrality—differences in the robustness to the deleterious effects of mutations—in viral populations (Sidebar A). Two of Holland's former collaborators—J. Quer (Barcelona, Spain) and I. Novella (Toledo, OH, USA)—summarized their recent work. Quer discussed work on the evolution of HBV and HCV (hepatitis B and C viruses, respectively), describing some initial results on the identification of natural drug-resistant variants by massive nucleotide sequencing. Novella presented results on the distinction between fitness and adaptability of VSV mutants. Some VSV mutants seem to have a limited capacity for producing beneficial mutations, which results in poor adaptability. Therefore, selection might operate on viral fitness, adaptability and robustness.

Mutation rates do not seem to be a limiting step for the variation that can be observed in RNA viruses. Rather, tolerance to mutations seems to be an important restricting factor. This fact was illustrated by Billeter with respect to the tight packaging constraints of paramyxoviruses: each nucleocapsid protein is in contact with strictly six nucleotides, and tight ribonucleotide structures seem to limit the occurrence of mutations and prevent copy-choice-directed recombination, thereby contributing to the stability of inserts engineered into the measles virus genome. Paradoxically, the measles virus can occasionally be subject to cellular RNA-editing mechanisms that can generate heavily hypermutated versions of the viral genome, such as those found in the brains of patients afflicted with subacute sclerosing panencephalitis (Cattaneo *et al*, 1988).

The relevance of the mutant-spectrum composition and complexity in determining virus behaviour has been one of the most intriguing observations made about viral quasispecies in recent years. M. Vignuzzi (Paris, France) summarized his work with R. Andino and C. Cameron, and parallel studies performed by the group of K. Kirkegaard, on the remarkable attenuation shown by a poliovirus mutant that encodes a polymerase of higher copying fidelity than the wild type. Indeed, the mutant generated a narrower mutant spectrum than the wild-type virus, which was a trait that impaired its pathogenic potential in a mouse model (Pfeiffer & Kirkegaard, 2005; Vignuzzi *et al*, 2006). The possibility that a phenotype characterized by the restriction of population complexity could be exploited to design attenuated vaccines is under investigation. Ongoing studies of fidelity mutants have provided evidence of complementation among the components of a viral quasispecies *in vivo*. Such interactions within quasispecies populations can be either positive—as in the case of complementation—or negative—as in the case of interference by dominant-negative mutants—and render quasispecies integrated units of selection. The response of a quasispecies can also be influenced by the 'molecular memory' of past dominant genomes, as discussed by C. Briones (Madrid, Spain; Sidebar A). Such collective behaviour had been predicted by the quasispecies theory, albeit involving mechanisms different from those operating in real viruses. Negative interactions within the mutant spectrum might contribute to lethal mutagenesis through the interfering activity of a subclass of genomes that have been termed defectors. Internal cohesions or repulsions within quasispecies mark a crucial difference with respect to the classical Wright–Fisher models of mutation–selection equilibrium, and they should be considered as characteristics of quasispecies behaviour, in addition to high error rates.

Despite a relative dislike of the quasispecies concept among some population biologists, its integration with some classical concepts of population genetics has provided—and is still providing—new insights into virus population behaviour. One example is the operation of the competitive-exclusion principle (Sidebar A) in the course of superinfection by HCV *in vivo* after liver transplantation, which was presented by S. Ramírez (Barcelona, Spain). Another example is the evidence of Red Queen dynamics (Sidebar A) in the process of HAV (hepatitis A virus) readaptation to codon usage in order to modulate the kinetics of translation for fitness gain, which was discussed by R. Pintó (Barcelona, Spain).

Hepatitis C is one of the viral systems in which the quasispecies theory has gained importance over the past decades, although many open questions still remain. J.-M. Pawlotsky (Paris, France) discussed the implications of quasispecies distributions in HCV infections for virus transmission, infection and persistence, tissue compartmentalization and response to treatment. The quasispecies complexity might have an influence on the maintenance of HCV infection by facilitating virus escape, which is an important factor in the face of the host immune response. By contrast, during the establishment of chronic

infection, immunological factors—in particular an impairment of the cellular immune response—seem to be the dominant influence.

The relevance of in-depth nucleotide sequencing for the management of HCV infection in the coming era of the clinical application of new protease and polymerase inhibitors—several of which are at advanced stages of clinical trials—was also debated. HCV quasispecies complexity, disease progression and response to treatment were also discussed by J. C. Sáiz (Madrid, Spain). No differences in quasispecies parameters were observed according to the stage of liver disease (Franco *et al*, 2003). Similarly, the presence of a dominant genome did not correlate with the clinical outcome, although it correlated with a higher viral load, in one study (Mas *et al*, 2004). In addition, in a study in which patients were classified into non-responders and sustained responders to interferon plus ribavirin treatment, no significant differences in quasispecies complexity could be established among the three groups, although they differed in the rate of fixation of mutations (Puig-Basagoiti *et al*, 2005). Therefore, it is currently unclear whether, or how, quasispecies dynamics might affect disease outcome. The newly developed massive sequencing techniques should be of great help in characterizing the composition of mutant spectra, provided that computational methods can be applied to distinguish technical errors from authentic sample heterogeneity, as discussed by O. Zagordi (Zurich, Switzerland). This important issue is a bane to all those involved in what is known as deep or ultra-deep sequencing.

The biological effects of bottleneck events were discussed for several viral systems, including FMDV by Domingo, and HIV-1 (human immunodeficiency virus type 1) by C. López-Galíndez (Madrid, Spain), R. Lorenzo (Madrid, Spain) and J. J. López-Moya (Barcelona, Spain). Bottleneck events are relevant as promoters of genetic drift in viral evolution, for diversification within infected hosts and as mediators of fitness decrease in viral populations (Fig 1; Escarmís *et al*, 2006).

Heterogeneity in natural RNA virus populations is the norm, and this extends to small DNA viruses such as the circoviruses, which behave similarly to RNA viruses, as discussed by M. Cortey (Barcelona, Spain). The problem in evaluating population heterogeneity is to distinguish whether diversification is the result of short-term evolution, re-infection or even, in some cases, reactivation of latent viruses. Whatever its origin, it is not easy to plan preventive or therapeutic strategies to confront such diversity. One of the tenets of antiviral vaccine design is that they should stimulate a broad immune response; consequently, vaccines composed of attenuated or whole inactivated viruses should be more effective than peptide vaccines. However, F. Sobrino (Madrid, Spain) showed that multivalent dendrimeric peptides could be used to elicit a powerful antibody and cellular immune response, and to evoke protection against FMDV in pigs, which are encouraging observations for peptide-based vaccine designs.

The effect that HIV-1 variability has on disease outcome was an obvious focus of interest at the meeting. R. Paredes (Badalona, Spain) documented that treatment failure is associated with the presence of drug-resistant minority mutants—not detectable in the consensus sequence—that were identified by an allele-specific PCR. M.A. Martínez (Badalona, Spain) reported an analysis of the fitness landscapes of HIV-1 protease quasispecies, which were rugged and in which the most abundant sequence was not the one with the highest fitness. Remarkably, each of the three quasispecies that Martinez analysed had distinct fitness landscapes, despite similar overall diversity. A. Meyerhans (Saarbrucken, Germany) compared recombination events in HCV and HIV-1. By using a replicon designed to select HCV recombinants, his group has been able to quantify the recombination frequencies for HCV, and to compare them with the wealth of data already available for HIV-1. Interestingly, both intra-subtype and inter-subtype recombination events—which are frequent in HIV-1—seem to be rare in HCV. Nonetheless, as there are an average of 2×10^{10} HCV-infected cells per patient, it is estimated that 2×10^{6} recombination events can occur every 2–5 days. Therefore, recombination probably has a role in HCV drug-resistance, as is the case for HIV-1.

An important body of theoretical and experimental work projects quasispecies dynamics towards new arenas. S. Manrubia (Madrid, Spain) discussed the theoretical models that relate robustness, neutrality and adaptability, and the intricate connections between endogenous—for example, population size and mutation rate—and exogenous—environmental changes—influences that might render high neutrality detrimental. These models promise ample conceptual applications—including to real viruses in an epidemiological setting—and predict movements in neutral networks to access new phenotypes. However, they are based on unavoidable simplifications such as the traditional approach of using the secondary structure of RNA as the phenotype. A remarkable example of phenotypic diversification was discussed by S. Ojosnegros (Madrid, Spain), who described the generation of two viral subclasses—termed colonizers and competitors—from a single FMDV clone after relatively short evolutionary times in cell culture, as would be predicted by some models of ecology.

Non-viral quasispecies

Are the main concepts and implications derived from the celebrated Qβ work and later developments restricted to viruses? The answer is certainly not. Population diversity and dynamics apply to cells, and particularly to cells that are rapidly evolving and have acquired a relatively autonomous behaviour. R. Solé (Barcelona, Spain) has applied the concept of quasispecies to cancer cells, considering them to be minimal replicators. Cancer instability is associated with point mutations and genome rearrangements, and competition among cellular forms contributes to tumorigenesis. There might be instability thresholds in cancer, which are counterparts of the quasispecies error threshold. The recognition of the interplay between cooperation and competition among the components of tumours might open new approaches to limit the adaptability of cancer cells.

Weissmann discussed some new and intriguing results that might shed light on the nature of PrPsc (prion protein) strain variation and selection, drawing interesting parallels to the world of viral quasispecies. The way in which prion strains are defined has remained a mystery for a long time. A recently developed cell-based assay—known as the CPA (cell panel assay)—allows discrimination between prion strains and is now being used to investigate whether 'mutant' versions of prions exist in the form of 'biased quasi-populations', akin to minority components in viral quasispecies. The exciting possibility that a prion population might undergo mutation and selection based on different conformational states should soon be resolved through application of the CPA (Klohn *et al*, 2003).

The fact that physiologically relevant cellular deaminases are clearly able to hyperedit RNA genomes, DNA genomes or retroviral genomes might be telling us much more than just that. In closing the meeting, S. Wain-Hobson (Paris, France) turned the logic around: hyperedited viral genomes can be seen as surrogate markers for the genetic editing of host RNA and DNA. This is well established

for the adenosine deamination of measles virus RNA and host-cell messenger RNAs, and for the cytidine deamination of some cellular messenger RNAs, which can be either highly specific or devastating—that is, highly damaging for the maintenance of genetic information. When it comes to the single-stranded DNA cytidine deaminases, it seems that, as no mechanism is perfect—something the three laws of thermodynamics told us long ago—they might occasionally turn on host-cell DNA. The obvious corollary is cancer development. Although it is not yet widely accepted beyond the realms of virology, Wain-Hobson suggested that there probably is far more genetic editing that hitherto believed (Petit *et al*, 2009).

The Barcelona celebration was a reflection of not only how concepts are sometimes generated fortuitously, but also how a purely theoretical development—in this case the quasispecies theory—can spark new experimental research that pervades different biological processes.

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